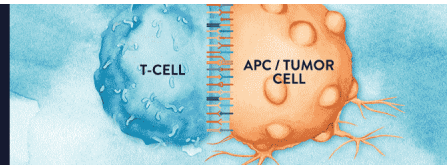


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Initial Characterization of the Vitamin D Binding Protein (Gc-Globulin) Binding Site on the Neutrophil Plasma Membrane: Evidence for a Chondroitin Sulfate Proteoglycan¹

Stephen J. DiMartino and Richard R. Kew²

The vitamin D binding protein (DBP) is a multifunctional plasma protein that can modulate certain immune and inflammatory responses. The diverse cellular functions of DBP appear to require cell surface binding to mediate these processes. Numerous reports have detected DBP bound to the surface of several cell types and would support the concept of a cell surface binding site for DBP. However, direct evidence for such a molecule has been lacking and essentially nothing is known about its basic biochemical properties. In the present study, radioiodinated DBP was used as a probe to characterize biochemically the neutrophil DBP binding site. Radiolabeled DBP binds to and remains associated with the plasma membrane and is not degraded. Quantitation of DBP binding to either intact cells or purified plasma membranes showed nonsaturable (linear) binding with positive cooperativity, possibly suggesting DBP oligomer formation. Solubilization of cell bound ¹²⁵I-DBP with various nonionic and zwitterionic detergents demonstrated that DBP binds to a membrane macromolecule that partitions to the detergent insoluble fraction. Moreover, this molecule does not associate with the cytoskeleton. Cross-linking of radiolabeled DBP bound to plasma membranes increased the amount of protein that partitioned to the insoluble fraction, and analysis of these complexes by SDS-PAGE revealed that they may be very large since they did not enter the gel. Finally, treatment of plasma membranes with either proteases or chondroitinase ABC completely abrogated membrane binding of DBP, suggesting that the protein binds to a chondroitin sulfate proteoglycan. *The Journal of Immunology*, 1999, 163: 2135–2142.

The vitamin D binding protein (DBP),³ also known as Gc-globulin, is a multifunctional plasma protein that can bind several diverse ligands (1, 2). DBP is a member of the albumin and α -fetoprotein gene family and shares considerable amino acid homology with these proteins (3, 4). The protein is synthesized predominantly by hepatocytes and circulates in blood at a concentration of 6–7 μ M as a single polypeptide chain with a molecular mass of ~56 kDa (1). DBP functions to transport vitamin D sterols, acts as a scavenger protein to clear extracellular G-actin released from necrotic cells, and a deglycosylated form of DBP has been shown to be a potent macrophage activating factor (5). In addition, we and others have demonstrated that purified DBP can significantly enhance the chemotactic activity (i.e., cochemotactic activity) of C5a and C5a des Arg (C5-derived peptides) for human (6–10) and bovine neutrophils (11). DBP has also been shown to augment monocyte and fibroblast chemotaxis to C5-derived peptides (12, 13). However, the chemotactic enhancing

properties of DBP appear to be restricted to C5a/C5a des Arg, since this protein cannot enhance the chemotactic activity of formylated peptides, IL-8, leukotriene B₄, or platelet activating factor (6–11). Although DBP appears to be a physiologically important regulator of leukocyte chemotactic activity for activated complement, the mechanism of chemotactic enhancement is not yet known.

Numerous investigators have reported a cell-associated form of DBP in many cell types, including neutrophils (14–27). Cell-associated DBP is not a novel cellular form but rather plasma-derived DBP bound to the cell surface (22). DBP bound to the plasma membrane of neutrophils appears to play an essential role in enhancing chemotaxis to C5-derived peptides (28, 29). It has been demonstrated that the binding and uptake of DBP by neutrophils is temporally correlated with generation of cochemotactic activity, and prevention of cellular uptake by an Ab (DBP pretreated with polyclonal anti-DBP) also precludes formation of cochemotactic activity (29). Moreover, the binding of DBP to its putative cell surface binding site may be important for several other functions of the protein, including delivery of vitamin D sterols, clearance of DBP-G actin complexes, and activation of macrophages. Therefore, a better understanding of the biochemical characteristics of its cell surface binding site is necessary to understand the diverse cellular functions of DBP. The goal of the present paper was to characterize the biochemical properties of the putative DBP binding site on human neutrophils. While a great deal of evidence supports the existence of such a cell surface molecule, essentially nothing is known about its basic biochemical properties. In this report, we demonstrate that radioiodinated DBP binds to a molecule on the neutrophil plasma membrane that partitions to the detergent insoluble fraction but is not associated with the cytoskeleton. Moreover, the cross-linked DBP-binding site complex does not enter an SDS-PAGE gel, suggesting that the complex may be very large. The unusual binding and biochemical characteristics of

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³ Abbreviations used in this paper: DBP, vitamin D binding protein; GAG, glycosaminoglycan; DSP, dithiobis(succinimidylpropionate); DTSSP, 3,3'-dithiobis (sulfo-succinimidylpropionate); sulfo-EGS, ethylene glycol bis(sulfo-succinimidylsuccinate); TX, Triton X; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; BCA, bicinchoninic acid.

the binding site suggests that DBP may interact with large glycosaminoglycan (GAG)-containing macromolecules. Enzymatic treatment of plasma membranes confirmed that DBP binds to a chondroitin sulfate proteoglycan.

Materials and Methods

Reagents

Purified human DBP was purchased from Biodesign International (Kennebunkport, ME). PMSF and 1,10-phenanthroline were purchased from Sigma (St. Louis, MO). All other protease inhibitors were purchased from Bachem (Torrance, CA). The chemical cross-linkers, dithiobis(succinimidylpropionate) (DSP), 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP), and ethylene glycol bis(sulfosuccinimidylsuccinate) (sulfoEGS) were purchased from Pierce (Rockford, IL). Cytochalasin D and the detergents Triton X-100 (TX-100), Triton X-114 (TX-114), digitonin, *n*-octyl *B*-D-glucopyranoside (octyl glucoside), and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) were purchased from Sigma. Heparinase I (from *Flavobacterium heparinum*), heparinase III (heparitinase, from *F. heparinum*), chondroitinase (ABC from *Proteus vulgaris*, AC from *F. heparinum*), trypsin, and chymotrypsin were purchased from Sigma. Sialidase (neuraminidase) was purchased from Boehringer Mannheim (Indianapolis, IN). Human leukocyte elastase and cathepsin G were obtained from Elastin Products (Owensville, MO). The bicinchoninic acid (BCA) and microBCA protein assay kits were obtained from Pierce.

Isolation of human neutrophils

Neutrophils were isolated from the venous blood of healthy, medication-free, paid volunteers (who gave informed consent) using a standard three-step isolation procedure described previously (29).

Radioiodination of DBP

Purified DBP (200–400 μ g) was incubated with one Iodobead (Pierce) and 1 mCi of Na¹²⁵I (DuPont-NEN, Wilmington, DE) for 5 min. The reaction was terminated by removing the solution from the Iodobead. Free Na¹²⁵I was separated from ¹²⁵I-DBP by gel filtration on a PD-10 (Sephadex G-25; Pharmacia LKB, Piscataway, NJ) desalting column. The ¹²⁵I-DBP was concentrated using a Centricon 30 microconcentrator (m.w. cutoff, 30 kDa; Millipore, Bedford, MA). TCA at 10% was used to determine the percentage of protein-associated counts. Radioiodinated DBP preparations generally had >99% of the total counts precipitable with 10% TCA. Finally, protein concentration was measured using the microBCA assay, and an aliquot was examined by SDS-PAGE and autoradiography to determine its purity.

Disruption and subcellular fractionation of neutrophils

Purified neutrophils were suspended at 75–100 \times 10⁶ cells/ml in ice-cold disruption buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 10 mM HEPES, 1 mM ATP (pH 7.4), containing 1 mM PMSF, 2 mM 1,10-phenanthroline, 100 μ M E-64, and 25 μ M pepstatin A), then added to a prechilled nitrogen cavitation bomb (Parr Instrument Company, Moline, IL). The cells were equilibrated to 350 psi with constant stirring for 20 min and then collected in a dropwise fashion into 125 mM EGTA (pH 7.4) to achieve a final concentration of 1.25 mM EGTA (30). Nuclei and unbroken cells were removed by centrifugation at 800 \times g for 10 min at 4°C. Prechilled (4°C) Percoll gradients (Pharmacia LKB) were prepared by progressively underlaying 2.2 ml of each increasing density (1.05, 1.09, and 1.12 g/ml) in a 14 \times 89-mm Ultra-Clear centrifuge tube (Beckman Instruments, Palo Alto, CA). The supernatant from the nitrogen cavitation (~3 ml) was layered on top of the Percoll gradient and centrifuged at 50,000 \times g for 50 min in a prechilled SW41 swinging bucket rotor (Beckman) (31). Fractions were collected by aspiration using sterile, glass Pasteur pipettes. Percoll was removed from the fractions by centrifugation at 100,000 \times g for 60 min at 4°C.

The purified neutrophil subcellular fractions were resuspended in equal volumes of HBSS, the total protein content then was measured using the microBCA assay, and the purity of each subcellular fraction was assessed using marker assays for plasma membranes and each granule population (30). The purity of the plasma membrane and azurophil granule preparations were evaluated using quantitative assays for alkaline phosphatase and myeloperoxidase activities, respectively. The purity of gelatinase granules was assessed using gelatin zymography. A Western blot for lactoferrin was used to evaluate the purity of specific granules. Plasma membrane fractions contained 97 \pm 3.2% (*n* = 11) of the total alkaline phosphatase activity. The azurophil granules possessed 72 \pm 6.3% (*n* = 9) of the total myeloperoxidase (MPO) activity with balance of the MPO activity found in the

specific granule fraction. The nonquantitative gelatinolytic and lactoferrin assays revealed that the bulk of the gelatinase activity was in the gelatinase granule fraction, whereas the majority of the lactoferrin was localized to the specific granule fraction.

Quantitative binding assay

Neutrophils (10⁷ cells/sample) or purified neutrophil plasma membranes (5 μ g total protein/sample) were incubated with ¹²⁵I-DBP in HBSS containing 0.1% BSA (assay buffer) in a total volume of 100 μ l. Samples were incubated on ice (2°C) for 60 min. After the incubation period, the samples were applied to a Millipore vacuum filtration manifold to separate free from bound radiolabeled ligand. Samples were separated using 25-mm, circular Durapore type VV filters with 0.1- μ m pore size (Millipore). The filters were presoaked with 1% BSA (in HBSS) to block nonspecific binding of protein. After vacuum separation, the material bound to the filters was washed four times with 1 ml of ice-cold assay buffer, then the filters counted in a gamma counter for the amount of bound ¹²⁵I-DBP. All samples were assayed in triplicate or quadruplicate. For each concentration of radiolabeled DBP, a buffer control was included that contained no cells or membranes. Radioactivity that bound to filters under these conditions was considered the background value and was subtracted from the appropriate samples.

Preparation of neutrophil detergent lysates

Detergent lysates of neutrophils were prepared by adding 100 μ l of 1% detergent, 50 mM HEPES (pH 7.4) containing 20 mM benzamidine, 10 mM EDTA, 10 mM Na₃N, as well as the following inhibitors added fresh immediately before lysis: 2 mM PMSF, 2 mM 1,10-phenanthroline, 0.5 mM E-64, 0.2 mM 3,4-dichloroisocoumarin, 0.1 mM leupeptin, and 0.1 mM pepstatin. Lysates were vortexed thoroughly until all particulate matter was solubilized (usually 5–10 s) and then placed at 37°C for 60 min. The detergent insoluble material was then pelleted by centrifuging the lysates in a microfuge for 10 min at 15,000 \times g at 4°C.

PAGE and autoradiography

Samples were separated by SDS-PAGE using the discontinuous buffer system described by Laemmli (32). Samples were prepared for electrophoresis by boiling (100°C) for 7 min with an equal volume of electrophoresis sample buffer (0.125 M Tris (pH 6.8), 20% glycerol, 4% SDS) containing 0.2 M DTT as the reducing agent and separated on a homogenous 10% polyacrylamide separating gel with a 4% polyacrylamide stacking gel. After electrophoresis, the gels were stained, destained, dried, and exposed to x-ray film at –80°C.

Results

Previous work from our laboratory has demonstrated that the binding of DBP to neutrophils is essential for the chemotaxis enhancement of C5a (28, 29). Moreover, analysis of neutrophil-associated DBP by immunofluorescence suggested that the protein was localized to the cell surface (19). However, it was not clear whether neutrophils internalized and processed DBP or whether the protein remained bound to the cell surface. To determine the fate of DBP bound to neutrophils, cells were allowed to bind ¹²⁵I-DBP for 45 min at 37°C, washed, then were disrupted by nitrogen cavitation, and the subcellular fractions were isolated and counted for radioactivity. Fig. 1 demonstrates that > 87% of the bound DBP associated with the plasma membrane fraction. Subsequent analysis of the plasma membranes showed that >98% of the radioactivity was precipitable with 10% TCA. In addition, SDS-PAGE revealed that the plasma membrane-bound radioiodinated DBP migrated at 56 kDa, the same as purified native DBP, indicating that the protein was not degraded (data not shown). These results demonstrate that DBP binds to, and remains associated with, the plasma membrane.

The binding characteristics of ¹²⁵I-DBP to intact neutrophils (10⁷ cells) and neutrophil plasma membranes (5 μ g) were investigated using increasing concentrations of radiolabeled ligand. Fig. 2 shows that the binding to either intact cells (Fig. 2A) or neutrophil plasma membranes (Fig. 2B) is linear (nonsaturable) up to 4 μ M (Fig. 2A) or 5 μ M (Fig. 2B) radiolabeled DBP. These experiments would suggest that the affinity of DBP for its binding site

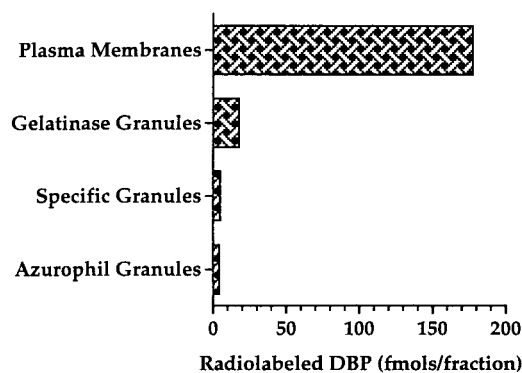


FIGURE 1. DBP associates with the neutrophil plasma membrane. Purified neutrophils from one donor were incubated in HBSS with 100 nM ^{125}I -DBP for 45 min at 37°C. Cells were washed with ice-cold HBSS, then resuspended in disruption buffer at 75×10^6 cells/ml. After disruption in a nitrogen cavitation bomb, the subcellular fractions were separated using Percoll gradients. The fractions were isolated, washed in cold disruption buffer, and counted for radioactivity. Data is expressed as fmols of ^{125}I -DBP associated with each fraction.

is very low. However, extensive washing of the plasma membranes in a detergent (1% TX-100) or high salt (1 M NaCl)-containing buffer did not remove the previously bound DBP (data not shown). Moreover, 100-fold molar excess of unlabeled DBP could not displace previously bound radiolabeled protein (data not

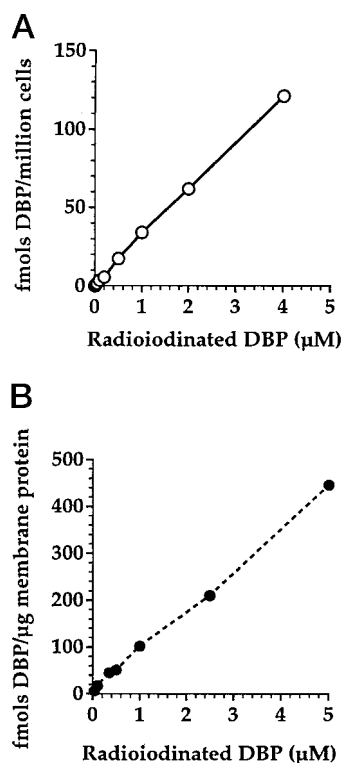


FIGURE 2. Binding of radiolabeled DBP to neutrophils and neutrophil plasma membranes. Purified neutrophils (10^7) or neutrophil plasma membranes ($5 \mu\text{g}$ total protein) were incubated in HBSS containing 0.1% BSA with the indicated concentrations of ^{125}I -DBP in a total volume of 0.1 ml. Samples were incubated on an ice slurry (2°C) for 60 min, and free ligand was then separated from the bound using a vacuum filtration device. Filters were counted for radioactivity, and the data are presented as fmols radiolabeled DBP bound either per 10^6 neutrophils or per μg membrane protein ($n = 2-4$).

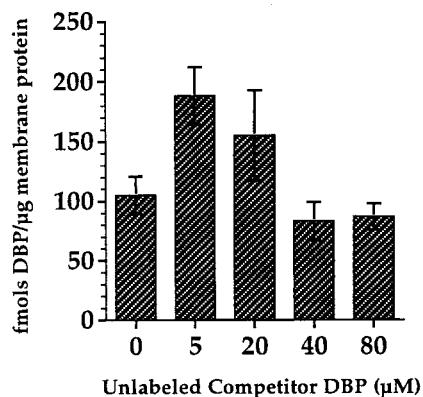


FIGURE 3. Competitive binding of radiolabeled DBP to neutrophil plasma membranes. Purified neutrophil plasma membranes ($5 \mu\text{g}$) were incubated in HBSS containing 0.1% BSA with $1 \mu\text{M}$ ^{125}I -DBP plus the indicated concentrations of unlabeled DBP in a total volume of 0.1 ml. Samples were incubated on an ice slurry (2°C) for 60 min, and free ligand was then separated from the bound using a vacuum filtration device. Filters were counted for radioactivity, and the data are presented as fmols radiolabeled DBP bound per μg membrane protein ($n = 2$).

shown), suggesting that the binding was relatively tight. In addition, the nonsaturable binding probably was not due to a large number of binding sites, since a 10-fold reduction in the concentration of membranes ($0.5 \mu\text{g}$) or cells (10^6) produced almost identical linear binding curves (data not shown). Another possibility to explain nonsaturable binding is that the binding site induces oligomerization of the DBP. Therefore, to examine this potential mechanism, a competitive binding assay was performed where radiolabeled protein was bound to membranes in the presence of increasing concentrations of unlabeled DBP (Fig. 3). If the interaction of DBP with its binding site is 1 mol per mol, then a competitive binding assay should demonstrate decreasing levels of radiolabel bound with increasing amounts of unlabeled competitor protein. Fig. 3 clearly shows that unlabeled protein increases the amount of radiolabeled DBP bound to the membranes at the lower concentrations, indicating some form of positive cooperativity, perhaps oligomerization of the ligand. These data demonstrate that DBP binding to the neutrophil plasma membrane is nonsaturable and suggest that the binding site induces oligomerization of DBP.

Our previous attempts to identify the DBP binding site by immunoprecipitating unlabeled DBP bound to surface-labeled cells were largely unsuccessful. In retrospect, this was not surprising given the unusual binding characteristics (Figs. 2 and 3). Therefore, a less selective approach was chosen to elucidate the biochemical properties of the DBP binding site on neutrophils. Since nonionic and zwitterionic detergents have been used extensively for the solubilization and characterization of numerous membrane proteins, we compared several detergents for their ability to solubilize membrane-bound DBP. Five nondenaturing detergents with different structures were employed: the nonionic detergents TX-100, TX-114, octyl glucoside, digitonin, and the zwitterionic detergent CHAPS. In addition, a complete protease inhibitor mixture was added to each detergent lysis buffer immediately before solubilization. For each detergent, solubilization revealed that ~50% of the total cell-associated radiolabeled DBP remained with the insoluble fraction (Fig. 4). Additional washes (total of six) of the insoluble material in 1% detergent liberated <10% of the previously bound DBP. Radioiodinated DBP, by itself, added to detergent was essentially 100% detergent soluble. Solubilization on ice (2°C), instead of 37°C, yielded no detectable differences in either

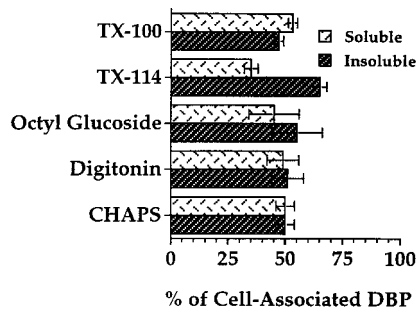


FIGURE 4. DBP associates with the insoluble fraction of neutrophils lysed using nonionic detergents. Purified neutrophils were incubated in HBSS with 100 nM ^{125}I -DBP for 60 min at 37°C. After washing, the cells were lysed using one of five detergents as follows: (a) 0.5% TX-114 for 1 h on ice, (b) 1% TX-100 for 1 h at 37°C, (c) 1% octyl glucoside for 1 h on ice, (d) 1% digitonin for 1 h at 37°C, (e) 1% CHAPS for 1 h at 37°C. All lysates included a protease inhibitor mixture (see *Materials and Methods*). The soluble and insoluble fractions were separated ($15,000 \times g$ for 10 min) and counted for radioactivity. Data are expressed as the percent of total cell associated radioactivity found in each fraction. The results represent the mean + SD of three to six separate experiments performed in duplicate using neutrophils from different donors.

the percent soluble vs insoluble DBP or in the m.w. of radioiodinated DBP by SDS-PAGE (data not shown). The percent distribution of the radiolabeled DBP was not altered if, during the pre-incubation step, neutrophils were either kept at 2°C, or in buffer lacking calcium and magnesium ions (PBS), or pretreated with phosphatidylinositol specific phospholipase C (5 U/ml) (data not shown). Furthermore, conditions designed to break divalent cation-dependent (10 mM EDTA) or ionic interactions (1 M NaCl) had no effect on the distribution of DBP to the insoluble fraction (Fig. 5). However, denaturing conditions (2% SDS or 0.1 M sodium carbonate (pH 11.0)) completely solubilized the protein (Fig. 5). These data indicate that a large percentage of DBP associates with the insoluble fraction of neutrophils solubilized under non-denaturing conditions.

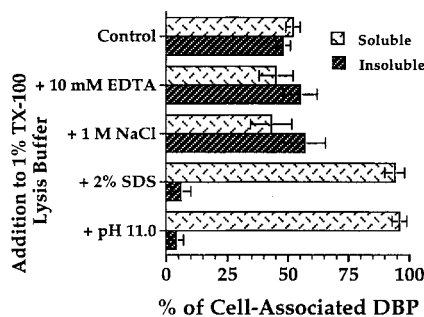


FIGURE 5. SDS and sodium carbonate (pH 11.0) can release DBP from the TX-100-insoluble fraction of a neutrophil lysate. Purified neutrophils were incubated in HBSS with 100 nM ^{125}I -DBP for 60 min at 37°C. After washing, the cells were lysed in 1% TX-100, which contained one of the following: (a) 10 mM EDTA; (b) 1 M NaCl; (c) 2% SDS; (d) 0.1 M sodium carbonate (pH 11.0). For the samples containing SDS and sodium carbonate, DNase I was added to decrease viscosity of the lysate (due to DNA released from the nuclei), which facilitated separation of the soluble and insoluble fractions. DNase I had no effect on the distribution of DBP (data not shown). Data are expressed as the percent of total cell associated radioactivity found in each fraction. The results represent the mean + SD of two to four separate experiments performed in duplicate using neutrophils from different donors.

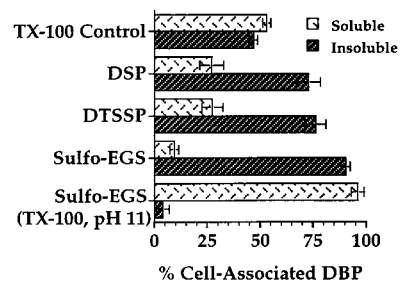


FIGURE 6. Homobifunctional chemical cross-linkers increase the amount of DBP associated with the TX-100 insoluble fraction of neutrophil lysates. Purified neutrophils were incubated in HBSS with 100 nM radioiodinated DBP for 30 min at 37°C. The cells were washed, then placed on an ice slurry and incubated with 1 mM DSP, 1 mM DTSSP, 1 mM sulfo-EGS, or no cross-linker for 30 min. After cross-linking, the reaction was terminated by addition of Tris (pH 8.0; final concentration of 10 mM). The cells were then washed once in ice-cold HBSS and lysed for 1 h at 37°C using either 1% TX-100 (pH 7.4) or 1% TX-100 in 0.1 M sodium carbonate (pH 11.0). The soluble and insoluble fractions were separated and counted for radioactivity. Data are expressed as the percent of total cell-associated radioactivity found in each fraction. The results represent the mean + SD of two to five separate experiments using neutrophils from different donors.

The detergent-insoluble cell fraction contains many cell surface proteins, as well as cytoskeletal elements (33). Accordingly, to determine whether DBP was interacting with cytoskeletal proteins indirectly via cell surface molecules, neutrophils first were pre-treated with 10 μM cytochalasin D (to disrupt the actin cytoskeleton) before incubation with radiolabeled DBP and lysis in TX-100. Cytochalasin D treatment had no effect on the partitioning of DBP to the insoluble fraction (52% TX-100 insoluble, 48% soluble). Moreover, radiolabeled DBP also associated with the detergent-insoluble fraction after binding to neutrophil plasma membranes (56% TX-100 insoluble, 44% soluble), which lack a cytoskeleton. These data suggest that DBP binds to a cell surface molecule(s) that is insoluble for reasons other than association with the cytoskeleton.

Partitioning of DBP to the detergent-insoluble fraction could be an artifact of solubilization, i.e., solubilization might disrupt the DBP-binding site complex, and the liberated DBP could then bind artifactually to a molecule that partitions with the insoluble fraction. To confirm that, before cell lysis, DBP associates with a molecule that will partition to the detergent-insoluble fraction, DBP was cross-linked to neutrophils using: DSP (membrane permeable cross-linker), DTSSP (water-soluble analogue of DSP), and sulfo-EGS (water soluble, nonmembrane permeable). Neutrophils were incubated with radiolabeled DBP at 37°C followed by cross-linking for 30 min on ice. Treatment with all cross-linkers resulted in a significant accumulation of DBP in the TX-100 insoluble fraction, as compared with the control sample (Fig. 6). However, >95% of the DBP-binding site complex was solubilized when sulfo-EGS cross-linked cells were lysed in 1% TX-100 containing 0.1 M sodium carbonate (pH 11.0) (Fig. 6). This indicates that the covalent cross-links are not responsible for causing the observed insolubility, since sodium carbonate does not break the covalent cross-links of sulfo-EGS. In addition, this data demonstrates that the cross-linked DBP-binding site complex can be solubilized using denaturing conditions. This experiment demonstrates that DBP binds to a molecule(s) that is destined to partition to the insoluble fraction following detergent solubilization.

DBP was cross-linked to intact neutrophils to determine the m.w. of its binding site. Both the detergent-soluble and -insoluble

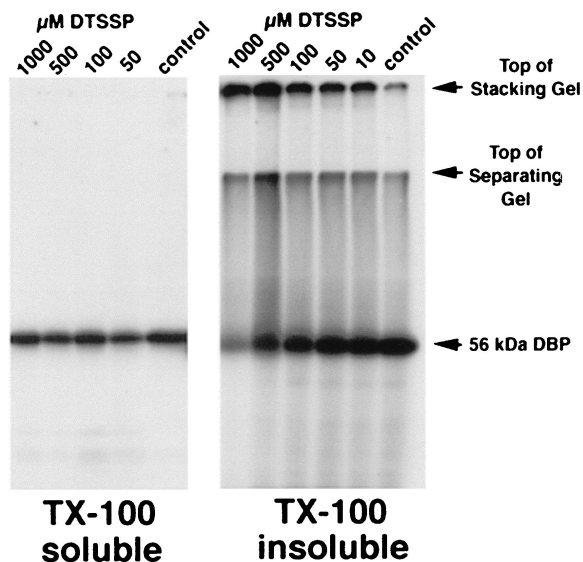


FIGURE 7. SDS-PAGE and autoradiography of DBP cross-linked to neutrophils. Purified neutrophils were incubated in HBSS with 250 nM ^{125}I -DBP for 30 min at 37°C. The cells were then washed and resuspended in ice-cold HBSS. Various concentrations of DTSSP were added, and the mixtures were incubated for 30 min on ice. The cross-linking reaction was stopped by adding Tris (pH 8.0) to a final concentration of 10 mM and then incubating on ice for 15 min. The cells were washed and then lysed in 1% TX-100 for 1 h at 37°C. The soluble and insoluble fractions were separated and counted for radioactivity. Next, samples were boiled in SDS buffer and ~25,000 cpm were loaded into each lane of a 10% SDS-PAGE gel with a 4% stacking gel. All samples shown are nonreduced. The results essentially were identical for the lipid soluble cross-linker DSP (data not shown).

fractions of DBP cross-linked to neutrophils were analyzed by SDS-PAGE and autoradiography (Fig. 7). In the soluble fractions, DBP was observed only at its native m.w. (56 kDa). However, in the insoluble fractions, DBP was observed in two large complexes, one that did not enter the stacking gel and another that appeared at the top of the separating gel. Interestingly, the control sample showed that DBP could participate in these complexes even in the absence of cross-linker, although this was a minor component (Fig. 7). When DTSSP concentrations greater than 1000 μM were used, all the radioiodinated DBP shifted to the two large complexes at the top of the gel (data not shown). The cross-linked complexes probably were not due to random collisional interactions, since it has been shown that random collisional cross-links are observed only when cross-linking is performed at elevated temperatures and high protein concentrations (34). Furthermore, cross-linking of DBP, in the absence of cells, generated no intermolecular complexes, despite being performed at a higher protein concentration (2 μM) and cross-linking temperature (37°C) (data not shown). These data demonstrate that, on neutrophils, DBP interacts with a large detergent-insoluble complex that does not enter an SDS-PAGE gel.

The unusual binding and biochemical characteristics of the binding site suggest that DBP may interact with large GAG-containing macromolecules. To address this possibility directly, intact neutrophils were treated with either proteases (pronase or trypsin, 1 mg/ml) or a mixture of GAG lyases (heparinase, heparitinase, and chondroitinase ABC, each at 1 U/ml) for 30 min at 37°C. Either treatment of neutrophils significantly reduced subsequent DBP binding to ~60% of control levels. This enzymatic approach was investigated further using purified neutrophil plasma membranes and the quantitative binding assay utilized above. Fig. 8A

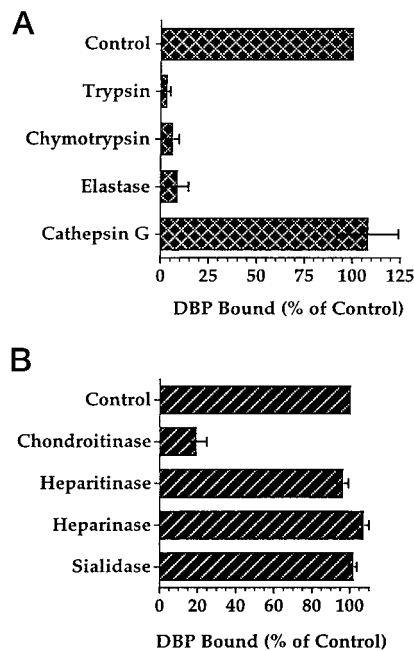


FIGURE 8. Effect of enzymatic treatment on the binding of DBP to plasma membranes. *A*, Protease treatment, 10 μg of plasma membranes were incubated in HBSS for 20 min at 37°C with either buffer alone or one of the following proteases: trypsin (1 mg/ml), chymotrypsin (1 mg/ml), elastase (1 μM), or cathepsin G (1 μM). *B*, GAG lyase treatment, 10 μg of plasma membranes were incubated in PBS (for heparinase, heparitinase, and sialidase) or 50 mM Tris, 60 mM sodium acetate (pH 8.0; for chondroitinase ABC) for 20 min at 37°C with buffer alone or one of the following: heparinase (5 U/ml), heparitinase (5 U/ml), sialidase (2 U/ml), or chondroitinase ABC (5 U/ml). Following this incubation, the membranes were pelleted at $14,000 \times g$ for 20 min at 4°C, and then were resuspended in HBSS 0.1% BSA containing 100 nM ^{125}I -DBP and were incubated for 1 h on ice. The samples were then filtered and washed as described in *Materials and Methods*. Filters were counted for radioactivity and the blank values subtracted from each sample value. All buffer controls (HBSS, PBS, Tris) had identical blank values. Data is expressed as fmols DBP bound as a percent of the control values. Data represents the mean + SEM of three to six separate experiments using membranes isolated from three different donors.

demonstrates that treatment of membranes with the broad specificity proteases, trypsin and chymotrypsin, completely eliminated subsequent DBP binding to membranes. Treatment of plasma membranes with the two major neutrophil proteases, elastase and cathepsin G, revealed that elastase treatment reduced DBP binding by >90%, whereas cathepsin G had no significant effect (Fig. 8A). Treatment of plasma membranes with GAG lyases showed that only chondroitinase ABC could significantly diminish subsequent DBP binding (Fig. 8B). In addition, DBP binding to membranes was also significantly reduced following treatment with chondroitinase AC, indicating that chondroitin sulfate B (dermatan sulfate) probably is not involved in DBP binding to neutrophils. These results indicate that DBP binds to a chondroitin sulfate proteoglycan on the neutrophil plasma membrane.

Discussion

Numerous investigators have reported that DBP associates with the plasma membrane of human blood monocytes, lymphocytes, and neutrophils (14–19), the B lymphoblastoid cell line Raji (20), HL-60 and U937 cell lines (21, 22), human placental trophoblast and smooth muscle cells (23, 24), rat pancreatic acinar cells (25), porcine kidney tubule cells (26), and human sperm (27). With few

exceptions (i.e., hepatocytes) it has been shown that the great majority of cells do not synthesize DBP found on their cell surface, but incorporate it from the extracellular fluids (22). Thus, DBP appears to bind to many diverse types of nucleated cells. However, one possible exception is RBC, since we have been unable to detect binding of radioiodinated DBP to purified, washed human RBC (R. Kew, unpublished observations). Despite the large number of reports that support the existence of a cell surface binding site for DBP, direct evidence for such a molecule has been lacking. In this paper, biochemical data characterizing the properties of the neutrophil DBP binding site are presented. The results show: 1) DBP binds to and remains associated with the plasma membrane and is not degraded; 2) DBP binding to either intact cells or plasma membranes is nonsaturable binding and shows positive cooperativity, possibly suggesting DBP oligomer formation; 3) DBP binds to an integral membrane macromolecule that partitions to the detergent insoluble fraction, and this molecule does not associate with the cytoskeleton; 4) cross-linking of membranes increases the amount of DBP that partitions to the insoluble fraction, and SDS-PAGE analysis revealed that these complexes may be very large since they did not enter the gel. 5) Enzymatic treatment of plasma membranes showed that DBP binds to a chondroitin sulfate proteoglycan. These properties of the DBP binding site clearly indicate that the molecule is not a classic high-affinity, single binding site receptor, but may be one or more heterogenous GAG-containing macromolecules.

The binding of radioiodinated DBP to neutrophils (Fig. 2) (29) is remarkably similar, in terms of kinetics and total amount per million cells, to the ^{125}I -DBP binding to porcine kidney tubule cells (26) and the B lymphoblastoid cell line Raji (20) reported previously. Moreover, DBP bound to porcine kidney tubule cells could not be displaced by 100-fold molar excess of unlabeled ligand (26), similar to what was reported herein. The two previous reports also showed that cell-associated DBP was degraded as evidenced by a high percentage of TCA soluble counts (20, 26). In contrast, we observed that neutrophils do not degrade ^{125}I -DBP and have often found that DBP is remarkably resistant to neutrophil-mediated proteolysis, despite the fact that these cells are often referred to as "bags of proteases." We have observed that several cell types (neutrophils, U937 cells, HL-60 cells) can hydrolyze the ^{125}I label (depending on the type of iodination procedure employed) without degrading DBP, thus producing a false positive reading by TCA precipitation or SDS-PAGE and autoradiography (R. Kew et al., unpublished observations). Nevertheless, the similarities in binding suggest that many cell types may possess a common cell surface binding site for DBP. A dissociation constant to measure the relative affinity of DBP for its binding site could not be obtained by Scatchard analysis because there was no saturation point in the binding isotherm (Fig. 2). However, the nonsaturable nature of DBP binding may indicate an alternative binding mechanism, such as an adsorptive process, as suggested previously (26).

Neutrophil lysates, from cells preincubated with radiolabeled DBP, demonstrated that the binding site for DBP partitions equally between the detergent-soluble and -insoluble fractions. Non-ionic detergents vary in their ability to solubilize membrane macromolecules. For example, the formyl peptide receptor is only partially soluble (50%) in TX-100 but is completely soluble in octyl glucoside (35). However, in the present study, the DBP binding site was $\sim 50\%$ soluble in all detergents examined. The detergent-soluble fraction probably represents free DBP that was released from cells during solubilization. Purified DBP added to TX-100 was 100% detergent soluble. Furthermore, cell-bound DBP that partitioned to the soluble fraction was always observed at its native m.w. (56 kDa) when cross-linked cells or membranes were ana-

lyzed by either SDS-PAGE or gel filtration chromatography, indicating that the detergent-soluble DBP did not form a complex. In contrast, the detergent-insoluble fraction appears to represent DBP bound to its cell surface binding site. The association with the insoluble fraction appears to be relatively tight because 1 M NaCl, low pH, and 100-fold molar excess of unlabeled protein could not dissociate radioiodinated DBP from the insoluble fraction. Moreover, cross-linkers greatly enhanced the amount of DBP in the insoluble fraction, probably by preventing the dissociation of DBP from its binding site. Denaturing conditions, such as 2% SDS or 0.1 M sodium carbonate (pH 11) solubilized 94% or 97%, respectively, of the DBP bound to cells. Thus, the DBP-binding site complex could be solubilized with anionic detergents or extreme alkaline conditions. However, a small percentage of cell-associated DBP remained insoluble (SDS-resistant) when neutrophils were solubilized with SDS under control (non cross-linked) conditions (Figs. 5 and 7). It is interesting to note that $6 \pm 4\%$ of DBP was insoluble in SDS (Fig. 5), whereas $\sim 8.5\%$ (determined by densitometry) of the cell-associated DBP was SDS-resistant in Fig. 7 (control lane). Although SDS can solubilize the majority of cell-associated DBP, a small percentage (5–10%) remains insoluble, and cross-linking increases this fraction (Fig. 7). SDS-resistant (insoluble) material would not be able to enter an SDS-PAGE gel and would appear at the top of the stacking gel (Fig. 7).

Nonionic detergents interact with membrane lipids and the hydrophobic domains of proteins (33). While they are quite effective at solubilizing membrane proteins and lipids, nonionic detergents are ineffective at breaking charge-dependent interactions and associate poorly with very hydrophilic molecules (33). Characterization of the detergent-insoluble cell fraction has shown that the cytoskeleton (a complex of hydrophilic proteins) is the major fraction remaining from cells solubilized with nonionic detergents (36, 37). Furthermore, some cell surface molecules that interact with the actin cytoskeleton have also been shown to distribute to the detergent-insoluble fraction. For example, the integrins, which are known to interact directly with the cytoskeleton, partition with the detergent-insoluble fraction (38). In neutrophils, β_2 integrins translocate to the TX-100 insoluble fraction after cells are stimulated with TNF- α (39). Likewise, in platelets, glycoprotein IIb-IIIa integrin becomes incorporated into the detergent-insoluble fraction upon platelet activation (40). For both models, receptor association with the insoluble fraction is reduced by pretreating the cells with cytochalasins. In contrast, the hyaluronan receptor (CD44) associates with the TX-100 insoluble fraction of cells, but not the actin cytoskeleton, and detergent insolubility of CD44 is not affected by cytochalasin pretreatment (41). Therefore, although detergent insolubility often suggests a linkage with the cytoskeleton, this is not always the case, and other cytoskeleton-independent mechanisms have been described. The detergent insoluble fraction also appears to be distinct from detergent-resistant membranes (DRMs), originally described by Brown and Rose (42). DRMs are isolated following TX-100 solubilization of cells on ice (42, 43). In the present study, cells were lysed for 60 min at 37°C, and, under these conditions, DRMs are completely solubilized (42, 43).

Numerous heparan sulfate and chondroitin sulfate proteoglycans have been shown to associate with the insoluble fraction of cells lysed in non-ionic detergents (44–48). For syndecan-1, a proteoglycan containing both heparan sulfate and chondroitin sulfate, the cytoplasmic domain (which could potentially interact with the cytoskeleton) is not necessary for the observed TX-100 insolubility, nor is the insolubility affected by pretreatment of cells with cytochalasins (48). However, removal of the GAG chains results in complete detergent solubility (48). These observations suggest that interactions involving the anionic GAG chains are responsible for

excluding non-ionic detergent. Moreover, proteoglycans can self-aggregate, and it is thought that this hydrophilic, anionic micro-environment serves to exclude nonionic detergents resulting in the partitioning of these molecules to the detergent-insoluble fraction (49–51). Cell surface proteoglycans are an attractive candidate for the putative DBP binding site. They are a very diverse family of macromolecules that are widely expressed on the plasma membranes of many cell types, including neutrophils (52, 53). Proteoglycans bind a wide variety of ligands and, depending upon the individual proteoglycan, binding can be nonsaturable and may induce ligand oligomerization. In addition, proteoglycans generally partition to the TX-100 insoluble fraction. Because of the large number of anionic GAG chains, many proteoglycans bind SDS poorly and thus do not enter an SDS-PAGE gel. Interestingly, a recent report demonstrated that platelet factor 4 (PF-4) binds to a chondroitin sulfate proteoglycan on the neutrophil plasma membrane, and many of the binding characteristics of PF-4 were similar to those of DBP presented in this report (54).

The putative cell surface binding site for DBP has remained a mystery for many years, probably because of the unusual binding characteristics and the inability of several investigators to isolate a specific membrane macromolecule. The results reported herein suggest that DBP binds to a chondroitin sulfate proteoglycan. Large heterogenous macromolecules, such as cell surface proteoglycans, would certainly explain the unusual cell-binding characteristics of DBP. Definitive identification of the proteoglycan(s) on the neutrophil plasma membrane that bind DBP should help to delineate the mechanisms of C5a cochemotaxis. Furthermore, the neutrophil DBP binding site may also be expressed on a wide variety of cells. However, it is not known if DBP binds to a specific chondroitin sulfate proteoglycan or any GAG-containing macromolecule; additional studies are needed to answer these questions. The information provided in this paper should clarify and focus future investigations aimed at identifying the cellular DBP binding site, and ultimately yield a better understanding of the diverse cellular functions of DBP.

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